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STUDIES ON THE NATURALLY OCCURRING HEMAGGLUTININ IN THE COELOMIC FLUID OF AN ASCIDIAN

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Recent studies on the immunological response revealed that an early indication of the appearance of adaptive immunity was found in one of the oldest types of vertebrates, a cyclostome (Good and Papermaster, 1964). In invertebrates, although their exact mechanisms remain obscure, there are many immunelike phenomena, for example, hemolysis, hemagglutination, bacteriolysis and bacterioagglutination by coelomic fluid, and the recognition of "self" or "not-self" by phagocytic cells (Huff, 1940, Briggs, 1966). The question whether the immune systems of vertebrates developed from one of the immunelike phenomena of invertebrates has not yet been solved (Burnet, 1968).

Although natural hemagglutinin can be found in the coelomic fluid of many invertebrates, its chemical and biological properties have been poorly understood (Marchalonis and Edelman, 1968; Makay, Jenkin and Rowley, 1969; Acton, Bennett, Evans and Schrohenloher, 1969). The discrimination between "self" and "not-self" by phagocytes was also observed in many invertebrates (Cameron, 1932; Aub, Tieslau and Lankaster, 1963), but its mechanism remains to be analyzed.

Data presented in this paper indicate the presence of hemagglutinin in the coelomic fluid of ascidians, uniquely placed between invertebrates and vertebrates. We then discuss its properties in relation to those of vertebrates.

Materials and Methods

Two species of ascidians, *Styela plicata* (Lesueur) and *Halocynthia hilgendorfi* f. *ritteri* (Oka) were used. They were harvested at Noto Marine Laboratory in Ishikawa, Japan. The coelomic fluid was collected by cutting the test and mantle without injuring the internal organs. After removing the cells by centrifugation, the coelomic fluid was collected and stored at −15° C.

The coelomic fluid was dialyzed to saline of an appropriate concentration used for the erythrocyte preparation from each animal. The red blood cells (RBC) from various animals were centrifuged at $450 \times g$ and suspended in veronal buffered saline, pH 7.2. The final concentration of RBC was adjusted to 10% (v/v, packed cells). The number of RBC of 10% (v/v) per ml from various animals were as follows; mice ($C_3H(He)$), 1.04×10^9; guinea pig, 1.02×10^9; sheep, 4.07×10^9; rabbit, 8.3×10^9; rat, 1.5×10^9; *Crassius carassius*, 4.23×10^8; *Bufo vulgaris*, 1.03×10^8; *Matrix tigris*, 3.15×10^8. To 0.3 ml of the 2-fold serially diluted coelomic fluid, 0.05 ml of red blood cells was added. After mixing completely,

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the hemagglutinating plate (Tomiki) was incubated at 37° C for 1 hour and the hemagglutination was observed.

For absorption, packed erythrocyte washed several times with saline was added to an equal volume of coelomic fluid which had been previously dialyzed to the same saline. After incubation at 37° C for 1 hour with occasional shaking, the mixture was centrifuged and the supernatant was used for hemagglutinin test. The secondary and tertiary absorptions were also tested.

Sephadex G-100 or G-200 (Pharmacia) was equilibrated by 0.02 M phosphate buffered saline (pH 7.8). Before filtration, the coelomic fluid was heated at 100° C for 20 minutes, centrifuged and then dialyzed to phosphate buffered saline over night. For trypsin digestion, the reaction mixture used was as follows: 0.1 ml of 1–5 mg/ml trypsin (Difco, 1 : 250), 0.3 ml of dialyzed coelomic fluid, 0.1 ml of 0.2 M phosphate buffer pH 7.8. The reaction mixture was incubated for 30 minutes, 2 hours and 4 hours at 37° C.

Periodate treatment was done as follows: the dialyzed coelomic fluid was incubated at 25° C for 3 hours with the various concentrations of periodate which was adjusted to pH 5.4 with 2 N NaOH. Then the coelomic fluid was again dialyzed to saline and its hemagglutinic activity was estimated.

For the observation of phagocytosis, the coelomic fluid was poured out onto a cover glass by cutting the test and the mantle. After 10 minutes, the supernatant was discarded and coleomic cells adhering to the glass were used for observation. After washing with sea water several times the cells were fixed with glutaraldehyde at the final concentration of 1%. After the fixation had been carried out for about 1 hour, the preparation was washed several times by distilled water and then stained with Giemsa solution (× 10) for 20 minutes.

For other observations on phagocytosis, the coelomic fluid was poured out onto the plastic dish (Falcon) and after 10 minutes, the rabbit erythrocytes (fixed with 1% glutaraldehyde) were added. Twenty minutes later, the cells were fixed with 1% glutaraldehyde.

Results

The occurrence of hemagglutinin

Specificity of hemagglutinin. Rabbit erythrocytes were shown to aggregate when they were mixed with ascidians' coelomic fluid, even at a high dilution. The titer of hemagglutinin of *S. plicata* to rabbit erythrocyte was $+2^{13}$ and that of *H. hilgendorfi* was $+2^{12}$. The erythrocytes of fish, frog, and snake were not aggregated by the coelomic fluid of either *S. plicata* or *H. hilgendorfi*. The hemagglutination seems to be restricted to erythrocytes of mammals, such as mice, rats and rabbits. The erythrocytes of sheep and guinea pig, although these are mammals, did not aggregate in the presence of ascidians' coelomic fluid. There are some variations in the agglutinating activity among species of ascidians. The coelomic fluid of *H. hilgendorfi* aggregated rat erythrocytes (titer: $+2^{7}$) but did not aggregate that of *S. plicata*. Conversely, the coelomic fluid of *S. plicata* aggregated mouse erythrocytes (titer: $+2^{4}$) but did not aggregate that of *H. hilgendorfi*.

The absorption test was carried out to determine whether the hemagglutinin adhered to rabbit erythrocytes and disappeared from the supernatant as with mammalian antibodies or not Two milliliters of the coelomic fluid of *S. plicata* were mixed with an equal volume of packed rabbit erythrocytes (1.8×10^{10} cells). After incubation at 37° C for 1 hour, the mixture was centrifuged and the supernatant was used for the hemagglutinin test (first step). The secondary and tertiary absorptions were done by the same procedure. After the tertiary absorption, the supernatant no longer aggregated rabbit erythrocytes. As described later, the coelomic hemagglutinin did not change its activity during an hour of incubation at 37° C.

The specificity of hemagglutinin was studied by the absorption method (see Table I). Tests were performed to determine whether the hemagglutinin for rabbit erythrocyte was identical to those of other animals and whether the hemagglutinin for rat or mouse erythrocyte was identical to that for rabbit erythrocyte. Two ml of the coelomic fluid of *S. plicata* and *H. hilgendorfi* was mixed with an equal volume of packed erythrocytes. The number of packed cells of several animals per ml were as follows; rat, 1.3×10^{10}, mice (C_3H), 1.1×10^{10}, rabbit, 8.3×10^9. After incubation at 37° C for 1 hour, the mixture was centrifuged and the supernatant was used for hemagglutinin test (primary dilution, $\times 2$). The secondary (dilution, $\times 4$) absorption was done by the same procedure. The questions of whether the hemagglutinin of *H. hilgendorfi* is equally effective for the aggregation of rat and rabbit erythrocytes, and of whether the agglutinin against mouse erythrocytes is identical to the hemagglutinin to rabbit erythrocytes in *S. plicata*, are answered in Table I.

It is clear that the hemagglutinin to rabbit erythrocytes was eliminated through the absorption by mouse erythrocytes in *S. plicata* and the hemagglutinin to rabbit erythrocytes of *H. hilgendorfi* was also eliminated through the absorption by rat erythrocytes. Conversely, the absorption by rabbit erythrocytes eliminated the hemagglutinating activity to mouse erythrocytes in *S. plicata* and to rat erythrocytes in *H. hilgendorfi*. The hemagglutinin of each species of ascidian is thought to be homogeneous. However, the hemagglutinin of the two species of ascidians are not the same because the coelomic fluid of *S. plicata* could not aggregate rat erythrocytes which were aggregated by that of *H. hilgendorfi*. Moreover, the hemagglutinin of *S. plicata* to rabbit erythrocytes was not absorbed by rat erythrocytes and also that of *H. hilgendorfi* was not absorbed by mouse erythrocytes. Therefore, it may be assumed that the hemagglutinin of each of these two ascidians reacts to a different position (hapten) on the surface of the rabbit erythrocytes.

Chemical properties of hemagglutinin. Some chemical properties of hemagglutinin were studied by using the coelomic fluid of *Styela plicata*.

The coelomic fluid dialyzed to saline was incubated at 37° C for 5 hours in the buffer solutions of various pHs. After incubation, the reaction mixture was centrifuged, and after adjusting pH to 7.2 the supernatant was used for the hemagglutinin test. The stability of the hemagglutinin was very high in the region of neutral and alkaline pHs but low in strong acidic conditions (Table II).

The hemagglutinic activity was not affected by the overnight dialysis to physiological saline. The coelomic fluid of *S. plicata* was dialyzed to saline containing 0.01 M EDTA overnight, and its hemagglutinating activity was tested. The activity

TABLE I

Specificity of hemagglutinin. For further details, see text

Ascidian	Erythrocyte used for absorption	Titers of hemagglutinin to		
		Rabbit	Rat	Mouse
Halocynthia hilgendorfi	No absorption			
	Primary (×2)	$+10 \times 2^{7}$	$+10 \times 2^{4}$	–
	Secondary (×4)	$+10 \times 2^{6}$	$+10 \times 2^{3}$	–
	Rabbit erythrocyte			
	Primary (×2)	$+10 \times 2^{3}$	–	–
	Secondary (×4)	+10	–	–
	Rat erythrocyte			
	Primary (×2)	$+10 \times 2^{4}$	–	–
	Secondary (×4)	$+10 \times 2^{1}$	–	–
	Mouse erythrocyte			
	Primary (×2)	$+10 \times 2^{7}$	$+10 \times 2^{4}$	–
	Secondary (×4)	$+10 \times 2^{6}$	$+10 \times 2^{3}$	–
Styela plicata	No absorption			
	Primary (×2)	$+10 \times 2^{8}$	–	$+10 \times 2^{1}$
	Secondary (×4)	$+10 \times 2^{7}$	–	+10
	Rabbit erythrocyte			
	Primary (×2)	$+10 \times 2^{3}$	–	–
	Secondary (×4)	+10	–	–
	Mouse erythrocyte			
	Primary (×2)	$+10 \times 2^{4}$	–	–
	Secondary (×4)	$+10 \times 2^{3}$	–	–
	Rat erythrocyte			
	Primary (×2)	$+10 \times 2^{8}$	–	$+10 \times 2^{1}$
	Secondary (×4)	$+10 \times 2^{7}$	–	+10

TABLE II

pH stability of hemagglutinin. For further details see text.

pH	Buffer (0.05 M)	Titers of haemagglutinin
pH 1.9	KCl-HCl buffer	$+2^{6}$
pH 2.9	citrate buffer	$+2^{8}$
pH 4.3	citrate buffer	$+2^{8}$
pH 5.5	citrate buffer	$+2^{10}$
pH 6.0	citrate buffer	$+2^{12}$
pH 7.8	phosphate buffer	$+12^{12}$
pH 8.6	borate buffer	$+12^{12}$
pH 9.6	borate buffer	$+12^{12}$

did not change as a result of this dialysis. Nor did the addition of $CaCl_2$ (2 mM) or $MgCl_2$ (2 mM) change the activity. Nor indeed did the addition of $CaCl_2$ (2 mM) and $MgCl_2$ (2 mM) change its activity.

The coelomic fluid was incubated at 0° C, 37° C, 75° C and 100° C for 30 minutes. No change of activity was observed at these temperatures. Moreover, the coelomic fluid was heated at 140° C for 30 minutes in an autoclave, but the hemagglutinic activity was not changed. Therefore it seems that the hemagglutinin is not protein.

The hemagglutinin was digested by trypsin in order to ascertain whether it was protein or not. The coelomic fluid of *S. plicata* was incubated with trypsin (1:250, Difco) at 37° C for several hours. Then the reaction was stopped by boiling at 100° C for 10 minutes. The reaction mixture was centrifuged at 450 × *g* for 15 minutes and the supernatant was used for hemagglutinic activity. No change of activity was observed due to these procedures.

TABLE III

Effect of periodate. For further details, see text.

Species	Periodate (M)	Titers of hemagglutinin
Halocynthia hilgendorfi	0	$+10 \times 2^8$
	0.04	−
	0.02	−
	0.004	$+10 \times 2^5$
Styela plicata	0	$+10 \times 2^6$
	0.04	−
	0.02	−
	0.004	$+10 \times 2^2$

From the evidence previously described, such as its heat stability and trypsin-resistant properties the hemagglutinin is considered to be a polysaccharide or mucopolysaccharide. To confirm this possibility, periodate treatment was performed because it is an agent known to destroy saccharide by oxidation.

The coelomic fluid was treated with various concentrations of periodate and then dialyzed to physiological saline. The reaction mixture is as follows; 1 ml of the coelomic fluid that was dialyzed to saline overnight, 0.3 ml of 0.2 M citrate buffer, pH 5.4, 0.2 ml of various concentrated periodate which was adjusted to pH 5.4 with 2 M NaOH. After incubation at 25° C for 3 hours, the reaction mixture was dialyzed to saline overnight to remove periodate. After centrifugation hemagglutinic activity of the supernatant was measured. The hemagglutinic activity was completely destroyed by 0.02 M periodate as shown in Table III. Since the hemagglutinin incubated at these pH conditions without periodate did not change its activity at 25° C, the destruction of activity must be due to oxidation of saccharide by the periodate.

The hemagglutinin was not dialyzable at 5° C overnight. Moreover, when ammonium sulfate was added to the coelomic fluid up to 50% saturation level, hemagglutinin was found in the precipitate. The precipitate was insoluble in water but was soluble in 0.85% NaCl. These properties suggested that it is a high-

weight molecular substance. The rough molecular weight was estimated by the gel-filtration on Sephadex. The coelomic fluid was heated at 100° C for 15 minutes, then centrifuged to remove the precipitate and applied to Sephadex G-100 column. A typical elution pattern was shown in Figure 1. The same experiment was done using the Sephadex G-200 column. As shown in Figure 1, two peaks appeared, and hemagglutinic activity was found at both peaks when Sephadex G-200 was used. The coelomic fluid seems to contain two molecular species of hemagglutinin. Assuming that the hemagglutinin is polysaccharide, the molecular weight of the smaller one could lie between 150,000 and 800,000 and the larger one be over 800,000.

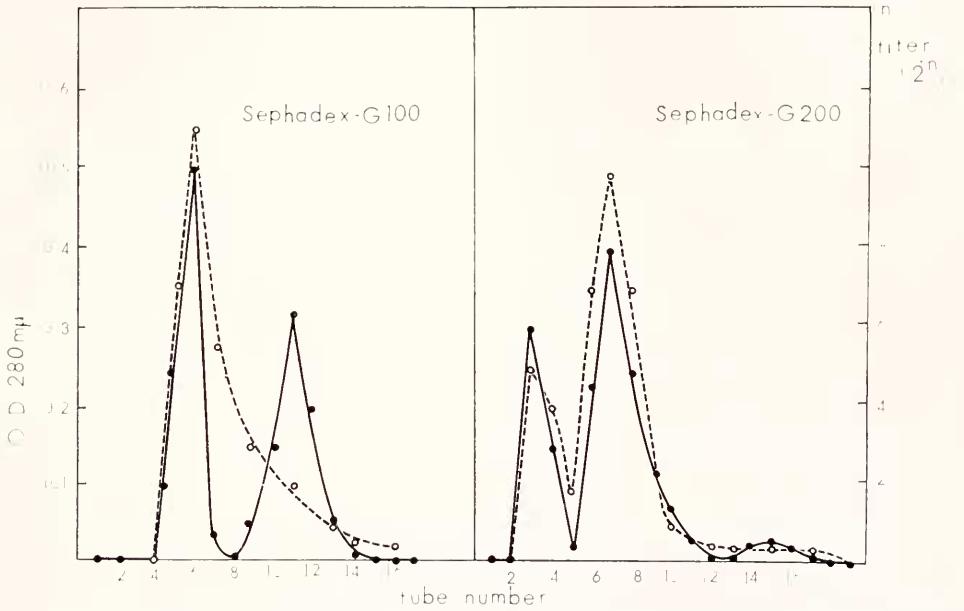

FIGURE 1. The elution pattern of the boiled coelomic fluid of *S. plicata* through sephadex G-100 and G-200 column. The coelomic fluid was boiled at 100° C for 15 minutes. After centrifugation, the supernatant ($O.D._{280\ m\mu} = 2.4$) was applied to the column. The elution buffer was phosphate buffered saline, pH 7.2 and the column size was 1.6×30 cm. One tube contains 6 ml of fractionated solution. Symbols used are: —●—●—, optical density at 280 mμ; - - ○ - - ○ - -, titers of hemagglutinin, 2^n.

Effect of the coelomic fluid on phagocytosis

The coelomic fluid of ascidians contained the hemagglutinin which was considered to be polysaccharide or mucopolysaccharide. It also contained a large number of coelomic cells. The fluid was examined to determine whether the ascidian's hemagglutinin could enhance phagocytosis of cells as with mammalian antibodies, although its chemical nature differed.

Identification of phagocytes in the coelomic fluid. After fixation and staining by Giemsa, the coelomic cells of *S. plicata* which have properties of adherence to the glass surface were observed. Four cell types of phagocytes were distinguished.

Fine-granular amoeboid cells were abundant and about 80% of coelomic cells are in this type. The cytoplasm contains many fine granules stained reddish with Giemsa which characterized these cells (Fig. 2, a). The size of the cells are of 5–10 μ. They show active amoeboid movement and phagocytosis (Fig. 2, e).

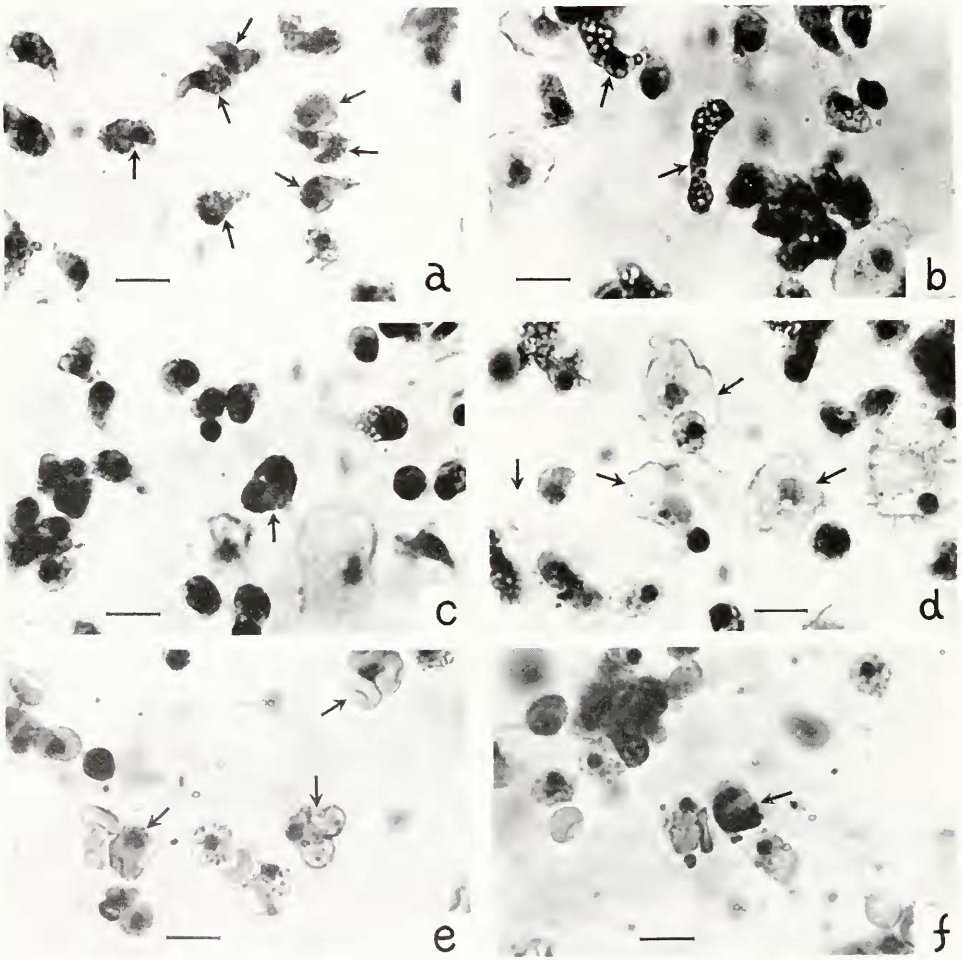

FIGURE 2. Phagocytic cells in the coelomic fluid of *Styela plicata*: (a) fine granular amoeboid cells; (b) granular amoeboid cells; (c) large basophilic cells; (d) vacuolated cells; (e) fine granular amoeboid cells which took in rabbit erythrocytes; (f) phagocytosis by granular amoeboid cell. The scale line indicates 10 μ.

The cells tend to aggregate when they come in contact with air. And when a toxic dose of dye or erythrocytes was injected into the coelomic cavity, these cells were also observed to aggregate in a sheet.

Granular amoeboid cells form about 15% of coelomic cells. They are large granules which are vitally stained with Nile blue. They showed a very active

amoeboid movement and very elongated forms were frequently observed in a smear preparation (Fig. 2, b). They showed phagocytosis (Fig. 2, f).

Large basophilic cells are difficult to find because they are about 1–2% of the total number of cells. The cell size was about 10–15 μ. The cytoplasm was stained with a characteristic grayish blue. One or two vacuoles were usually observed. The nucleus was relatively small and round, and had an eccentric position (Fig. 2, c). Cells usually do not contain erythroycte, but phagocytosis was occasionally observed.

Vacuolated cells are thin and elongated and have several vacuoles (Fig. 2, d). Phagocytic activity of these cells was very evident. The number of the cells varied depending on physiological conditions, for example, the cell number increased after starvation.

Effect of the coelomic fluid on phagocytosis. A test was made to determine whether the phagocytosis of the coelomic cells of *S. plicata* was affected by the presence of the coelomic fluid which contained the hemagglutinin.

TABLE IV

The Effect of the coelomic fluid on phagocytosis. For further details see text.

The concentration of coelomic fluid	% of phagocytic cells	s.e.m.
Sea water	49%	±1.8
$\frac{1}{5}$ coelomic fluid + $\frac{4}{5}$ s.w.	50%	±3.2
$\frac{1}{3}$ coelomic fluid + $\frac{2}{3}$ s.w.	48%	±1.8
$\frac{1}{2}$ coelomic fluid + $\frac{1}{2}$ s.w.	50%	±3.5
Coelomic fluid	49.5%	±1.7

The number of cells per milliliter of the coelomic fluid was estimated by haemocytometer. Some variations were observed between the different animals. The mean value of ten animals was 7.5×10^6 and the standard error of mean was ± 2.0.

One milliliter of coelomic fluid was poured on the dishes (d = 3.2 cm) and after ten minutes setting, the coelomic fluid was pooled and centrifuged at $450 \times g$. The supernatant was diluted with sea water at various concentrations ($\frac{1}{2}$ coelomic fluid + $\frac{1}{2}$ sea water; $\frac{1}{3}$ coelomic fluid + $\frac{2}{3}$ sea water; $\frac{1}{5}$ coelomic fluid + $\frac{4}{5}$ sea water) and added to the dishes. Fixed rabbit erythrocytes were added and after phagocytosis had proceeded for 20 minutes, the cells were fixed. The percentage of viable cells were determined by staining with 0.04% nigrocine. It showed over 95% after setting for 30 minutes. The number of coelomic cells which had taken an erythrocyte into their cytoplasm was counted. One ml of the coelomic fluid which contained the cells (7.0×10^6) was poured in to a plastic dish (d = 3.2 mm). 0.1 ml of red blood cells (1.4×10^6) was added. Twenty minutes later, cells were fixed and stained by Giemsa. 125×125 μ^2 each of phagocytic cells and non-phagocytic cells were counted. There were almost 50 cells in total per 125×125 μ^2. The cells were counted seven times. The results are shown in Table IV. It seems that the coelomic fluid was not essential for phagocytosis. To determine the effect of the coelomic fluid on phagocytosis more decisively, an experiment using the anti-coelomic fluid antibody is in progress.

However, from microscopical observations, it seemed that the coelomic cells aggregated with erythrocytes and with each other more actively in the presence of the coelomic fluid than in its absence. It also seemed that the coelomic fluid enhanced the adhesion of cells to glass surfaces.

Discussion

Present results showed that two ascidians possessed hemagglutinins for several mammalian erythrocytes in their coelomic fluids. These hemagglutinins are large molecules which can be absorbed by erythrocytes. These properties are similar to those of isohemagglutinin present in mammalian serum. But the hemagglutinin of ascidians is thought to be polysaccharide or mucopolysaccharide because it is very heat stable and destroyed by periodate.

The chemical properties of the hemagglutinin of ascidians are different from those of other invertebrates. Mackay *et al.* (1969) reported recently that the hemagglutinin of a crayfish was a protein which enhanced adhesion and phagocytosis of red cells by the phagocytic cells. The hemagglutinin of oysters was also reported as proteinaceous by Acton *et al.* (1969). The hemagglutinin of ascidians is similar to plant hemagglutinins rather than those of animals (Aub, *et al.*, 1963).

Burnet (1968) has recently suggested that such hemagglutinins may be forerunners of vertebrate immunoglobulins. However, the hemagglutinin of ascidians is not chemically related to vertebrate immunoglobulins. Moreover, structurally the hemagglutinin of oysters is demonstrably different from mammalian immunoglobulin (Acton *et al.*, 1969). The body fluids of ascidians possess heat labile bacterioagglutinin (unpublished data of the authors). There remains the possibility that such a protein in the coelomic fluid (other than hemagglutinin) is the ancestral precursor of immunoglobulin.

The biological functions of the hemagglutinin in ascidians still remain obscure, and more detailed experiments are required. The hemagglutinin is not essential for phagocytosis by coelomic cells in this experimental system, nor was it found that the hemagglutinin activated phagocytosis. However, this does not exclude the possibility that the hemagglutinin which became bonded to the cell surface of phagocytes could play an important role in the discrimination between self and not-self. An experiment using the anti-hemagglutinin is now in progress and should clarify the problem.

Summary

The occurrence of a natural hemagglutinin in the coelomic fluid of solitary ascidians, *Styela plicata* and *Halocynthia hilgendorfi* is reported. The hemagglutinin aggregated some mammalian erythrocytes and was absorbed by them. The hemagglutinins of the two ascidian species are specifically distinct.

The hemagglutinin of *Styela plicata* is a large molecule which is very heat stable, resistant to trypsin digestion, but is destroyed by periodate. These data suggest that the hemagglutinin is polysaccharide or mucopolysaccharide.

The hemagglutinin has no apparent opsonic effect, but it seems to play a role [illegible] adherence of cell-to-cell and cell-to-glass surface.

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